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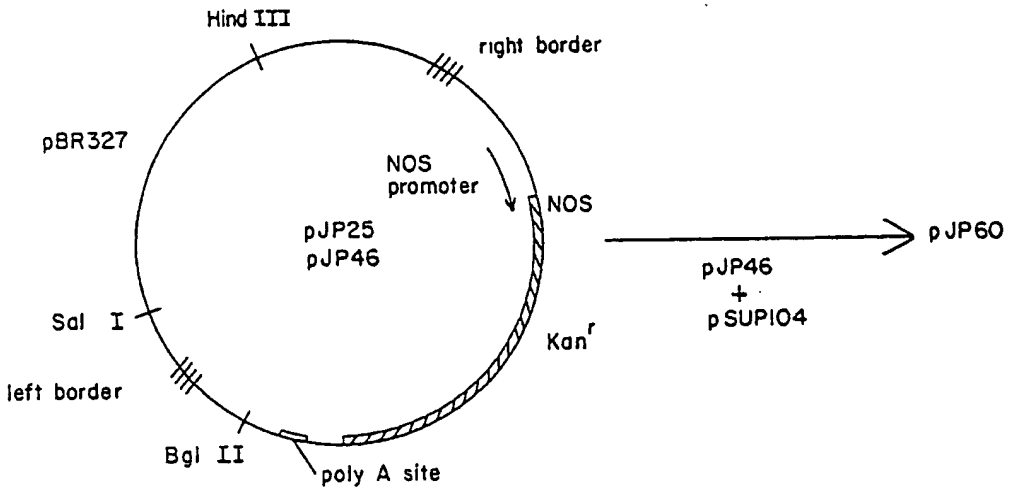

(57) Abstract

The productivity of modern agriculture is due, in large part, to the development of new and improved crop varieties through cross breeding of related species, which is however inherently imprecise, unpredictable, and slow. This invention permits the development of improved plants, without the limitations of classical breeding methods by providing a vector capable of being integrated into the chromosome of a plant protoplast cell. This vector, which is illustrated in Fig. 1, includes: 1) a DNA region encoding a selectable marker protein, transcription of this DNA region being under the control of a regulatory DNA sequence capable of effecting transcription in plant cells; 2) a polyadenylation site; 3) a desired heterologous gene, or a site for the insertion of such gene; and 4) DNA capable of causing the integration of the above DNA regions into a plant chromosome. The vector lacks sufficient Ti-derived DNA to cause tumor formation or the production of opines in the plant.

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PLANT TRANSFORMATION VECTOR

Background of the Invention

This application is a continuation-in-part of U.S. Pat. Appln. Serial No. 681,269 filed December 13, 1984.

This invention relates to the integration of heterologous genes into the genome of plant cells.

By the term "heterologous gene", we mean a gene which is a non-plant gene, a modified gene, a synthetic gene, a gene from a different plant strain or species, or a homologous gene from a different location in the plant's genome.

The productivity of modern agriculture is in large measure due to the development of improved crop varieties. This has been accomplished through classical plant breeding, where compatible varieties of crops are cross-bred to yield desired improvements. This method suffers from certain limitations, such as imprecision and unpredictability of results; long time frames for breeding programs; and the limitation of the available gene pool to those plants able to cross with each other (i.e., those of the same species).

Certain strains of Agrobacterium cause crown gall disease, a neoplastic transformation, in a broad range of dicotyledonous plants. Upon infection of a wound site, the Agrobacterium transfers at least a part of a large tumor-inducing (Ti) plasmid to the plant cell. A portion of this DNA, encoding the requisite functions for induction of tumor formation (T-DNA) is integrated into the plant cell genome. Also on the T-DNA are genes for the synthesis of opines, e.g., nopaline and octopine, which are produced by plant tissue containing integrated Ti-derived DNA. Ti plasmids are classified by the type of opine synthase

they carry; nopaline synthase (NOS) or octopine synthase (OS). Short sequences flanking the T-DNA (left and right border regions) are important for T-DNA integration.

Lawton et al. Hort. Sci. 19:10-12 (1984) discuss Ti plasmids as potential vectors for genetic engineering. An exogenous gene and selectable marker were integrated into plant cells using a recombinant Ti plasmid. No evidence of normal transcription of the introduced DNA was found.

Zambryski et al. EMBO 2:2143-2150 (1983) report the construction of a recombinant Ti plasmid, the tumorigenic portion of which had been deleted. Plant cells transformed with this vector grew normally and expressed NOS, which was left intact on the plasmid as a transformation marker. Genes, for tumor induction, were subsequently inserted into the recombinant Ti plasmid, and plant cells transformed with this plasmid expressed both NOS and the tumor phenotype.

Herrera-Estrella et al. EMBO 2:987-995 (1983) report the construction of a recombinant Ti plasmid containing an antibiotic resistance marker attached to T-DNA regulatory elements. Progeny cells from tobacco protoplast transfectants expressed antibiotic resistance, but yielded only tumor callus. Morphologically normal plants were not produced.

Horsch et al, Science 223:496-498 (1984) report the regeneration of morphologically normal, fertile plants from cells transformed with an OS Ti plasmid containing an inserted antibiotic resistance marker, and an intact NOS promoter, structural gene, and right border. The resulting plants expressed antibiotic resistance and produced nopaline.

Summary of the Invention

The present invention permits the development of improved plants, without the limitations of classical plant breeding methods, by making possible the direct manipulation of a plant's genome and the introduction into the genome of heterologous genes from any source. The invention thus eliminates the imprecision inherent in classical breeding methods, since any specific gene can be selected, for insertion into the plant's genome, to impart the desired functions. In addition, the invention allows for the development of modified plant varieties in much shorter time periods than is possible using traditional methods. Furthermore, the invention greatly broadens the gene pool from which useful genes can be selected, to include not only genes from other plants, but also genes from fungi, bacteria, and animals, as well as hybrid and synthetic genes.

Accordingly, the invention features, in one aspect, a vector capable of being integrated into the chromosome of a plant protoplast cell; the vector includes 1) a DNA region encoding a selectable marker protein, transcription of this DNA region being under the control of a regulatory DNA sequence capable of effecting transcription in plant cells; 2) a polyadenylation site; 3) a desired heterologous gene, or a site for the insertion thereof in the vector; and 4) DNA capable of causing the integration of (1), (2), and (3) into a chromosome of the plant cell, the vector lacking sufficient Ti-derived DNA to cause tumor formation in the plant or to cause the production of opines in the plant.

For transformation of plant cells in addition to protoplast cells, the invention features a vector having (1)-(4), above, and in addition includes DNA

sufficient to enable the vector to replicate in A. tumefaciens and to transform a plant cell when A. tumefaciens containing the vector is cocultivated with the plant cell; this vector also lacks sufficient tumorigenic DNA to cause tumor formation in the plant.

In preferred embodiments, the regulatory DNA is derived from a Ti plasmid encoding nopaline synthase or octopine synthase, in which case the vector is incapable of effecting the expression of nopaline or octopine, and the Ti border DNA is derived from a plasmid containing a gene encoding nopaline synthase or octopine synthase.

The vectors of the invention can be used to effect the normal expression in plants of desired proteins, with selection of transformants facilitated by a selectable marker protein, also expressed normally. Normal expression of the marker protein is effected by the regulatory sequence, preferably T-DNA-derived, and the polyadenylation site. Tumor formation is prevented by the exclusion of sufficient T-DNA to cause tumor formation. Furthermore, metabolic energy of the transformed plant is not wasted producing opines, i.e., octopine and nopaline, because the vectors of the invention are lacking all or a portion of the structural gene encoding nopaline synthase and octopine synthase.

The vector of the invention can be used in any suitable plant species. One class of suitable plants are dicotyledenous plants, e.g., tobacco, tomato, and petunia plants, in which Ti plasmids are known in nature to cause tumor formation. Other suitable plants include monocotyledenous plants capable of being transformed by Ti plasmids. Recently it has been shown that monocots, although they do not form A. tumefaciens-induced tumors, are capable of being transformed by the T-DNA of A. tumefasciens. This was demonstrated oy Hooykaas-Van

Slogteren et al. (1984) Nature 311, 763, who carried out the infection of Chlorophytum capense and Narcissus, both monocots, and detected in their tissues either nopaline or octopine, depending on the Ti plasmid present in the infecting A. tumefaciens.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

We now describe preferred embodiments of the invention, after briefly describing the drawings.

Drawings

Fig. 1 is a diagrammatic representation of two vectors of the invention, containing border regions derived from a NOS-encoding Ti plasmid.

Figs. 2-12 are diagramatic representations of intermediate constructions and steps leading to the vectors of Fig. 1.

Fig. 13 is a diagrammatic representation of a region of one of the vectors of claim 1, and Fig. 14 is a diagrammatic representation of the other.

Fig. 15 is a diagrammatic representation of two vectors of the invention, containing border regions derived from an OS-encoding Ti plasmid.

Figs. 16-23 are diagrammatic representations of intermediate constructions and steps leading to the vectors of Fig. 15.

Fig. 24 is a digrammatic representation of octopine TL border-containing DNA.

Fig. 25 is a diagrammatic representation of the synthetic 25 bp repeat sequence of the octopine TL right border.

Figs. 26-33 are diagrammatic representations of intermediate constructions and steps leading to the vectors of Fig. 33.

Fig. 34 is a diagrammatic representation of vectors containing a single synthetic border sequence.

Plasmid Components

As is mentioned above, the plasmids of the invention contain several essential DNA regions and sites, now discussed in greater detail.

Selectable Marker

Because transformation of plant cells with plasmids containing foreign genes is a relatively rare event, plasmids of the invention must contain a DNA region which encodes a selectable marker protein for identification of transformants. This marker protein can be any protein which can be expressed in plant cells and which enables the phenotypic identification of plant cells which express the protein. Preferred marker proteins are proteins which provide resistance to one or more antibiotics; currently most preferred is the protein aminoglycoside phosphotransferase, which inactivates antibiotics such as kanamycin, neomycin, and G418; transformants are those plant cells able to grow in the presence of antibiotic. Other examples include chloramphenicol acetyl transferase, which provides resistance to chloramphenicol; and dihydrofolate reductase, which provides resistance to methotrexate. Certain genes conferring herbicide resistance can also be used as selectable markers.

Polyadenylation Site

Eukaryotic (e.g., plant) messenger RNA's must be polyadenylated for efficient translation and processing. Polyadenylation requires a recognition site for polyadenylation enzymes near the 3' end of the DNA region encoding the selectable marker.

DNA Sequences for Integration

DNA derived from or substantially identical to

a border region of T-DNA is used to effect the integration of the plasmids of the invention into the chromosomes of host plant cells. Sequencing of several T-DNA border regions has revealed that each contains a direct repeat sequence 25 base pairs in length. One such sequence is sufficient to cause integration, and the vectors of the invention can include one or two such sequences, which may or may not be flanked by additional T-DNA border sequences.

Regulatory Sequences

Transcription of both the selectable marker gene and the desired heterologous gene, in order to lead to efficient expression, is preferably under the control of regulatory sequences normally expressed in plant cells, e.g., T-DNA promoters such as those for the NOS or OS genes. Examples of other suitable promoter sequences include those of the ribulose bisphosphate carboxylase small subunit gene, the nitrate reductase gene, and the glutamine synthase gene. Synthetic, engineered, or altered natural promoters can also be used. In some instances, it will be desirable to use promoters which are regulated, e.g., promoters active only at certain times in the plant's development. It is desirable that the plasmid of the invention contain less than the entire structural gene normally under the control of the each promoter used, to ensure that metabolic energy of the transformed plant is not wasted producing protein encoded by the structural gene.

Site for Desired Heterologous Gene

The site for the insertion of a desired heterologous gene can be any site at which an endonuclease can act to cut the plasmid for insertion of the desired heterologous gene. Preferably the site is unique in the plasmid, so that the endonuclease cuts the

plasmid only in the desired location.

Desired Heterologous Gene

The desired heterologous gene can be any gene which is capable of being expressed in the plant, and which encodes a protein which enhances a beneficial feature of the plant, or provides a new beneficial feature. Examples of such genes are those encoding resistance to herbicides, resistance to diseases such as tomato fusarium wilt, proteins which can be produced to improve the protein content or other nutritive value of the plant, and genes encoding biopesticides. Although the easiest modification to carry out is one involving a biochemical function which is controlled by a single protein, and is thus encoded by a single gene, more than one heterologous gene can be introduced, and the expression of such multiple genes controlled in a coordinate manner so as to introduce more complex biochemical functions into plants. Examples include multi-enzyme pathways, e.g. energy-generating reactions and biosynthetic pathways.

As mentioned above, transcription of the desired heterologous gene, like transcription of the selectable marker gene, is preferably under the control of a regulatory sequence normally expressed in plant cells. The fusion of the heterologous gene to the regulatory sequence can be carried out prior to the insertion of the heterologous gene into the vector, using conventional techniques. Alternatively, the gene can be inserted into the vector by itself, and the regulatory sequence inserted upstream from the gene separately, prior to or following the insertion of the gene, using conventional methods.

Plasmid Construction; Nopaline T-DNA Borders

Referring to Fig. 1, plasmids pJP25 and pJP46

contain the aminoglycoside phosphotransferase gene from Tn5, encoding resistance to the antibiotics kanamycin and G418 (the kan^r gene), under the transcriptional control of the NOS promoter; the nopaline T-DNA left and right border regions; a polyadenylation site; and a unique Bgl II site for insertion of a heterologous gene. These plasmids were constructed as follows (pJP25 and pJP46 differ only in that pJP25 contains a small amount of the NOS structural gene).

The first step was to insert the left and right T-DNA border regions from a NOS-encoding Ti plasmid into E. coli plasmid pBR327. Referring to Fig. 2, nopaline T-DNA, including the border regions, is present on the Ti plasmid carried by A. tumefaciens strain C58. Plasmid pGV0369 (Fig. 3) bears the left nopaline T-DNA border, and the Hind III 23 fragment (Fig. 2) of plasmid pGV0329 bears the nopaline T-DNA right border and the NOS structural gene.

Hind III fragment 23 was purified from a Hind III digest of pGV0329 and subcloned into pBR327. As shown in Fig. 4, two plasmids were derived from this construction: pJPS18 carries the NOS polyadenylation site, and pJPL19 carries the nopaline T-DNA right border region and the NOS promoter.

Plasmid pJPL19 (Fig. 5) was cut at the Nco I site and treated with Bal 31 to remove about 400 bp from each end. The DNA was then cut with BamH I, the ends filled in with DNA polymerase, a Sma I linker (CCCGGG) added, and the plasmid recircularized.

Clones were screened for Sma I and BamH I sites. Those with the Sma I site at a suitable distance from the Sac II site were sequenced to determine the exact position of the Sma I site. Plasmid pE61-8 (Fig. 6) was one of the plasmids obtained. pE61-8 contains

the nopaline T-DNA right border region and the NOS promoter.

The left border region was then inserted by cloning the Bgl II-Pst I fragment from pGV0369 into the BamH I site of pE61-8 to yield pE118 (Fig. 7), which contains the nopaline T-DNA right and left borders and NOS promoter, but still lacks the polyadenylation site and the kan^r gene.

Next, plasmid pVW90 (Fig. 8), carrying the kan^r gene, was constructed as follows. First, the Hind III-Sal I fragment from Tn5 was cloned in pBR327 to yield pVW60, as shown in Fig. 9. In pVW60, there is an ATG upstream from the SD sequence of the kan^r gene which could result in an incorrect translation start of the mRNA of this gene in a eukaryotic cell.

To remove this extra ATG sequence, pVW60 was cut at Bgl II and treated with Bal 31 to remove DNA up to the SD sequence. A Sal I linker was inserted and the plasmid religated. Clones resistant and sensitive to kanamycin were obtained. A number of these were sequenced to determine the position of the Sal I linker. The resulting clones, and the portions of the pre-kan^r DNA they contain, are shown in Fig. 10.

pVW61, 62, 63, 64, 65, 70 were all kanamycin resistant.

pVW80, 81, 82, 83, 84, 86, and 87 were all kanamycin sensitive.

pVW61, 63, and 65 still have the extra ATG so are not useful.

pVW62, 64, 70 have lost the extra ATG and have an SD sequence intact enough to give expression.

pVW81, 87, 82, 80, 83, 86, and 84 have lost the extra ATG and the SD sequence, pVW81, 87, and 82 have an intact kan^r gene, and pVW80, 83, 86 and 84 have lost

the first 7 codons of kan^r and can be used to make translational fusions with a gene expressed in plant cells.

Next, pVW86, above, was combined with the NOS polyadenylation site to form pVW90 (Fig. 8), as follows.

Plasmid pJPS18 (Fig. 11), discussed above, carries the 3' end of the NOS gene and the NOS polyadenylation recognition site. The 250 bp Sau 3A fragment, containing the polyadenylation site, was purified and ligated to Sma I linkers (TCCCGGGA); this created a Bgl II site at one end, enabling the orientation of the fragment to be determined. This fragment was cloned into the Sma I site of pACYC177 to produce pVW77. The Xma I fragment was cut out of pVW77 and cloned into the Xma I (Sma I) site at the 3' end of the kan^r gene of pVW86, to yield pVW90 (Fig. 8). (The pVW77 Xma I fragment was also cloned in pVW82, 84, and 87.)

Plasmid pVW90 was then combined with pE118 (Fig. 7, discussed above), as follows. pE118 was cut at the Sma I site and treated with Bal 31 to remove about 25-30 bp. The linearized plasmid was then blunt-end ligated with the Sal I fragment from pVW90 carrying the kan^r gene and polyadenylation site, to yield constructions having the structure shown in Fig. 12.

The fusion junction of several constructions was sequenced to obtain clones in which the kan^r gene was in frame with the ATG of the NOS gene. Two clones were obtained having in-frame gene fusions: pJP125 (Figs. 1 and 13) and pJP46 (Figs. 1 and 14). The Hind III-SalI fragment of plasmid pJP46 was cloned into plasmid pSUP104 in place of the existing Hind III-SalI fragment; pSUP104 is stable in A. tumefaciens and mobilizable from E. coli into Agrobacterium, to yield

plasmid pJP60 (Fig. 1). Plasmid pSUP104 is described in Simon et al. (1983) "Vector Plasmids for in vivo and in vitro Manipulations of Gram-Negative Bacteria" in Molecular Genetics of the Bacterial Plant Interaction (Puhler, ed., Berlin 1983), and is available from Agrigenetics Corporation.

Plasmid Construction; Octopine T-DNA Borders

Referring to Fig. 15, plasmids pVW125 and pVW126 contain the right and left border regions of the right T-DNA ("TR DNA") of the octopine T-DNA (the octopine T-DNA also includes left T-DNA, or "TL DNA"); the kan^r gene, under the transcriptional control of the NOS promoter; a polyadenylation site, and a unique Bgl II site for insertion of a heterologous gene. These plasmids were constructed as follows (pVW125 and pVW126 differ only in the orientation of the kan^r gene.)

The first step was to insert the left and right TR border regions into pBR327. Referring to Fig. 16, octopine TR DNA is present in A. tumefaciens strain B6. BamH I fragment 2 (16.5 kb) from the B6 Ti plasmid was cloned in pBR327 to yield pVW51 (Fig. 17).

The TR left border on pVW51, in an Eco RI-BamH I fragment, was cloned in pBR327 to yield pVW58 (Fig. 17). The TR right border, on an Eco RI fragment of pVW51, was then cloned into the Eco RI site of pVW58 to yield pVW75 (Fig. 18).

In order to clone the kan^r gene between the left and right TR borders, a unique restriction site had to be created between the two borders. To accomplish this, the Eco RI site between the left and right borders was converted to a Sma I site by a partial Eco RI digest, the sticky ends were filled in, and a Sma I linker was then inserted into one or the other Eco RI site, to yield pVW78 (Fig. 19), which bears a unique Sma

I site between the borders, and pVW79 (Fig. 20), which bears a unique EcoRI site between the borders.

Next, a plasmid was constructed in which the kan^r gene was under the transcriptional control of the NOS promoter. This was carried out generally as described above, in the construction of plasmids containing nopaline T-DNA border regions, although the resulting vectors differed slightly in structure from those constructed with the nopaline T-DNA borders. The first intermediate, containing the NOS promoter, is plasmid pE85-21 (Fig. 21), in which the Sma I site is 2 bp upstream from the NOS ATG. The kan^r gene-containing intermediate pVW93 (not shown) has a Sal I linker inserted 2 bp upstream from the ATG of the kan^r gene. To combine the two, pE85-21 was cut with Sma I and treated with Bal 31 to remove a few (5-10) bases. The Sal I fragment from pVW93 carrying the kan^r gene and the polyadenylation site was blunt end ligated into pE85-21 to yield pVW104 (Fig. 22).

The Bcl I-Bgl II fragment from pVW104 carries the NOS promoter, the kan^r gene, and the polyadenylation site. This fragment was purified and cloned into the Sma I site of pVW78 between the two TR borders, to yield pVW121 (Fig. 23).

Plasmid pVW121 carries the right TL border, which was deleted by cutting with Xho I and BamH I and religating. This yielded plasmids pVW125 and pVW126, which bear the kan^r gene oriented in different directions. BamHI linkers were ligated to the EcoRI-XhoI fragment of pVW125 and it was cloned into the BamHI site of plasmid pSUP104 to yield plasmid pVW130 (Fig. 15).

Plasmid Construction; Synthetic borders

Six T-DNA border regions have been sequenced,

i.e., borders of T-DNA (from a nopaline-producing Ti plasmid) and TL and TR DNA borders (from an octopine-producing Ti plasmid). All have a similar direct repeat of 25 base pairs at the border regions. The sequences of the borders derived from different T-DNA are not identical for all 25 base pairs.

The 25 base pair direct repeat of the octopine right TL border was synthesized, using conventional techniques, and is represented in Fig. 25. This synthesized DNA was cloned into the E. coli vector pUC8 (Fig. 26) to make it more easily accessible for further cloning. Sal I linkers were ligated on the ends of the synthetic DNA and it was cloned into the Sal I site of pUC8 to yield plasmid pVW132 (Fig. 27). Sma I linkers were also ligated on the synthetic DNA so that it could be cloned into the Sma I site of pUC8, yielding plasmids pVW133 (Fig. 28) and pVW134 (Fig. 29). In the Figures, the orientation of the border fragments relative to each other in the 3 plasmids is indicated by the arrow. The orientations of the border sequences in Ti plasmids are indicated in Figure 30, A. and B. Thus, the single vector was constructed such that the relative orientations of the synthetic border sequences was the same as the orientation of the border sequences in the naturally occurring Ti plasmid. The Hind III-BamH I fragment from pVW134 (Fig. 29), containing the synthetic border sequence in the correct orientation, was cloned into the Hind III-BamH I site of pVW132 (Fig. 27) yielding plasmid pVW136 (Fig. 31). A selectable marker was cloned into the BamH I site between the synthetic border sequences. The Bcl I-Bgl II fragment of plasmid pVW104 (Fig. 32), containing the kanamycin resistance gene and polyadenylation site attached to the nopaline synthase promoter, was cloned into the BamH I site of

plasmid pVW136 (Fig. 31) to yield plasmid pVW143 (Fig. 33A) and plasmid pVW142 (Fig. 33B). The two plasmids obtained differ only in the orientation of the Kan^r gene and its regulatory elements relative to the synthetic border fragments. BamHI linkers were ligated to the Hind III-Eco RI fragments of pVW143 and pVW142 (Fig 33 A-B) which were cloned into the BamHI site of plasmid pSUP104 to yield, respectively, plasmids pVW149 and pVW148, for mobilization to Agrobacterium tumefaciens strain LBA4404 for cocultivation experiments with plant cells.

In addition to the above-described plasmids (pVW142 and pVW143) containing two synthetic border sequences flanking the kanamycin resistance gene, plasmids have been constructed which contain only one such synthetic sequence; this single synthetic sequence is capable of effecting integration of the plasmid DNA into the host plant chromosome. One such plasmid, pVW144 (Fig. 34), was constructed as follows. The BclI-BglII fragment of plasmid pVW104 (Fig. 32), containing the kan^r gene and polyadenylation site attached to the nopaline synthase promoter, was cloned into the BamHI site of plasmid pVW133 (Fig. 28), containing one synthetic border sequence, to yield plasmid pVW140 (Fig. 34). The HindIII-EcoRI fragment of plasmid pVW140 was then cloned into plasmid pSUP104 to yield plasmid pVW144 (Fig. 34). Plasmid pVW145 (not shown) was derived from plasmids pVW104 (Fig. 32) and pVW134 (Fig. 29) in analogous fashion, and it differs from pVW144 only in the orientation of the single border sequence relative to the kanamycin resistance gene.

Plasmid Construction; Insertion of Heterologous Genes

Plasmids pJP25, pJP46, pVW125, and pVW126 all have a unique Bgl II site into which a desired

heterologous gene can be inserted, using conventional techniques.

There is a high probability that such a unique site is available on pVW142 and pVW143 as well. If such sites are not available, they can be created using standard methods.

Plant Transformation

The plasmids of the invention can be used to directly transform plant protoplasts, e.g., by packaging the plasmids in liposomes, using conventional techniques.

Alternatively, the plasmids can be used to transform plant protoplasts or non-protoplast cells, using a "binary" cocultivation technique with A. tumefaciens. To use this method, the pSUP104-derived plasmids (pJP60, pVW144, pVW130, pVW148, and pVW145) were transferred to an A. tumefaciens strain, e.g., LBA4404, which carries a Ti plasmid, pLBA4404, deleted of the T-DNA region, so that cocultivation does not result in plant tumor formation. (pLBA4404, and binary cocultivation, are described in Hoekema et al. (1983) Nature 303, 5913.) pLBA4404 does, however, retain the native Ti "vir" (for virulence) functions which are essential for the transfer of the hybrid plasmid of the invention from A. tumefaciens LBA4404 to the host plant cell. The A. tumefaciens containing the hybrid plasmid was then cocultivated with plant cells to insert the hybrid plasmid into the plant cells, where the plasmid DNA, by virtue of the natural or synthetic border DNA, integrated into the plant cell chromosome. Transformants were selected by means of the selectable marker.

Another plant transformation method, known as the "quick-dip" method, involves the dipping of plant

explants in the A. tumefaciens bacterial culture and then incubating the infected plant parts on medium that selects for kanamycin resistance and promotes shoot regeneration; the method is described in Horsch et al. (1985) Science 227, 1229.

Plant Regeneration

Following selection of transformants, the plant cells (protoplasts or other cells) are cultured under conditions effecting the regeneration of mature plants. Such methods are known, e.g., for the regeneration of tobacco plants from callus culture. The resulting mature plant, the cells of which contain integrated DNA of the vector of the invention, express the desired heterologous gene, fail to express the OS or NOS genes, and do not form tumors.

Deposits

Plasmids pJP46 and pVW125 have been deposited in the American Type Culture Collection, Rockville, MD, and given ATCC Accession Nos., respectively, of 39930 and 39929. Applicants' assignee, Biotechnica International, Inc., acknowledges its responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, and its responsibility to notify the ATCC of the issuance of such a patent, at which time the deposits will be made available to the public. Until that time the deposits will be made available to the Commissioner of Patents under the terms of 37 CFR §1.14 amd 35 USC §112.

Other Embodiments

Other embodiments are within the following claims. For example, the OS TL border regions (contained in A. tumefaciens strain ACH5), rather than the TR border regions, can be used, and the OS rather than NOS promoter used. A plasmid, pOTY8 (described in

Hirsch et al. (1984) Mol Gen. Genet. 195, 209; Fig. 24) contains the TL border regions. We have made subclones of pOTY8 containing the left TL border (pVW57) and right TL border (pVW59) have been made. pVW57 contains Hind III fragment 18 carrying the left border of TL cloned in pBR327, and pVW59 contains EcoRI fragment 24 carrying the right border of TL and the promoter of the OS gene cloned in pACYC184. Any of these border regions can be used to construct vectors in the same manner as described above.

CLAIMS

1. A vector capable of being integrated into the chromosome of a plant protoplast cell, said vector comprising

1) a DNA region encoding a selectable marker protein, transcription of said DNA region being under the control of a regulatory DNA sequence capable of effecting the transcription of said DNA region in said plant cell,

2) a polyadenylation site,

3) a desired heterologous gene, or a site for the insertion thereof in said vector, and

4) DNA capable of causing the integration of (1), (2), and (3) into said chromosome of said plant cell,

said vector lacking sufficient Ti-derived DNA to cause tumor formation in said plant or to cause the production of opines in said plant.

2. A vector capable of being integrated into the chromosome of a plant protoplast or non-protoplast cell, said vector comprising,

1) a DNA region encoding a selectable marker protein, transcription of said DNA region being under the control of a regulatory DNA sequence capable of effecting the transcription of said DNA region in said plant cell,

2) a polyadenylation site,

3) a desired heterologous gene, or a site for the insertion thereof in said vector,

4) DNA capable of causing the integration of (1), (2), and (3) into said chromosome of said plant cell, and

5) DNA sufficient to enable said vector to replicate in A. tumefaciens and to transform said plant

cell when said A. tumefaciens containing said vector is cocultivated with said plant cell,

said vector lacking sufficient Ti-derived DNA to cause tumor formation in said plant or to cause the production of opines in said plant.

3. The vector of claim 1 or claim 2 wherein said regulatory DNA sequence is derived from a Ti plasmid encoding nopaline synthase or octopine synthase, said vector being incapable of effecting the expression of nopaline synthase or octopine synthase.

4. The vector of claim 1 or 2, said integration-causing DNA being substantially identical to border T-DNA of a Ti plasmid containing a gene encoding nopaline synthase or octopine synthase.

5. The vector of claim 4, having ATCC Accession No. 39929.

6. The vector of claim 4, having ATCC Accession No. 39930.

7. A vector capable of being integrated into the chromosome of a plant protoplast cell, said vector comprising a desired heterologous gene, or a site for the insertion thereof in said vector, and DNA capable of causing the integration of said desired gene into said chromosome of said plant cell, said DNA comprising DNA substantially identical to border DNA of the left and right TR border regions of a Ti plasmid containing a gene encoding octopine synthase.

8. A plant cell transformed with the vector of any of claims 1, 2, or 7.

AMENDED CLAIMS

[received by the International Bureau on 2 May 1986 (02.05.86);
original claims 1-8 replaced by new claims 1-9 (2 pages)]

1. A vector capable of being integrated into the chromosome of a plant protoplast cell, said vector comprising

1) a DNA region encoding a selectable marker protein, transcription of said DNA region being under the control of a regulatory DNA sequence capable of effecting the transcription of said DNA region in said plant cell,

2) a polyadenylation site, and

3) DNA capable of causing the integration of (1) and (2) into said chromosome of said plant cell,

said vector lacking sufficient Ti-derived DNA to cause tumor formation in said plant or to cause the production of opines in said plant.

2. A vector capable of being integrated into the chromosome of a plant protoplast or non-protoplast cell, said vector comprising,

1) a DNA region encoding a selectable marker protein, transcription of said DNA region being under the control of a regulatory DNA sequence capable of effecting the transcription of said DNA region in said plant cell,

2) a polyadenylation site,

3) DNA capable of causing the integration of (1) and (2) into said chromosome of said plant cell, and

4) DNA sufficient to enable said vector to replicate in A. tumefaciens and to transform said plant cell when said A. tumefaciens containing said vector is cocultivated with said plant cell,

said vector lacking sufficient Ti-derived DNA to cause tumor formation in said plant or to cause the production of opines in said plant.

3. The vector of claim 1 or claim 2, further comprising a desired heterolgous gene, or a site for the insertion thereof in said vector.

4. The vector of claim 1 or claim 2 wherein said regulatory DNA sequence is derived from a Ti plasmid encoding nopaline synthase or octopine synthase, said vector being incapable of effecting the expression of nopaline synthase or octopine synthase.

5. The vector of claim 1 or 2, said integration-causing DNA being substantially identical to border T-DNA of a Ti plasmid containing a gene encoding nopaline synthase or octopine synthase.

6. The vector of claim 5, having ATCC Accession No. 39929.

7. The vector of claim 5, having ATCC Accession No. 39930.

8. A vector capable of being integrated into the chromosome of a plant protoplast cell, said vector comprising a desired heterologous gene, or a site for the insertion thereof in said vector, and DNA capable of causing the integration of said desired gene into said chromosome of said plant cell, said DNA comprising DNA substantially identical to border DNA of the left and right TR border regions of a Ti plasmid containing a gene encoding octopine synthase.

9. A plant cell transformed with the vector of any of claims 1, 2, or 8.

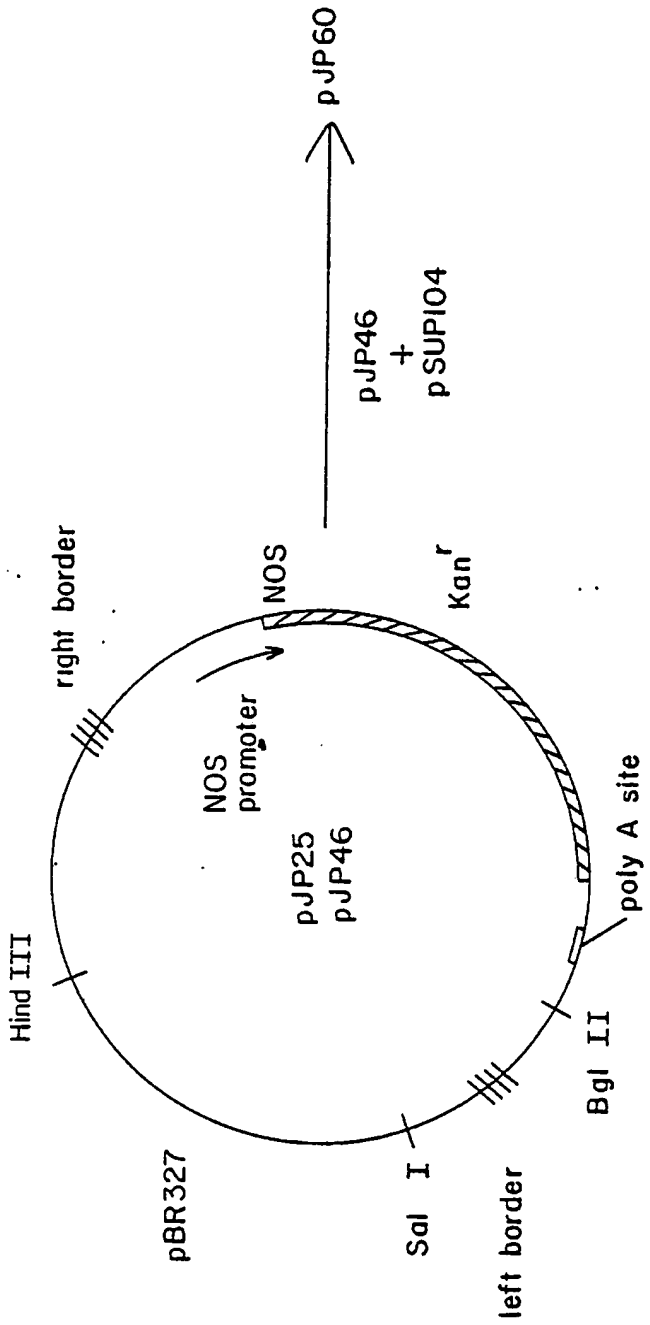


FIG 1

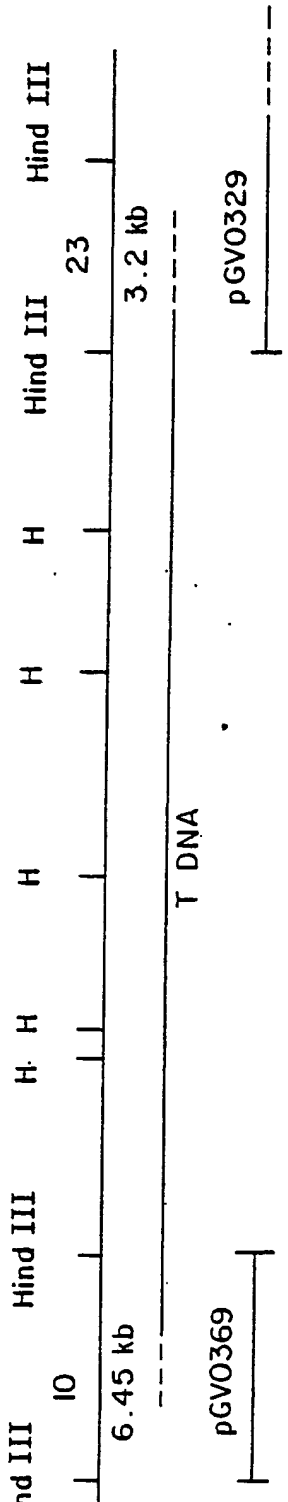


FIG 2

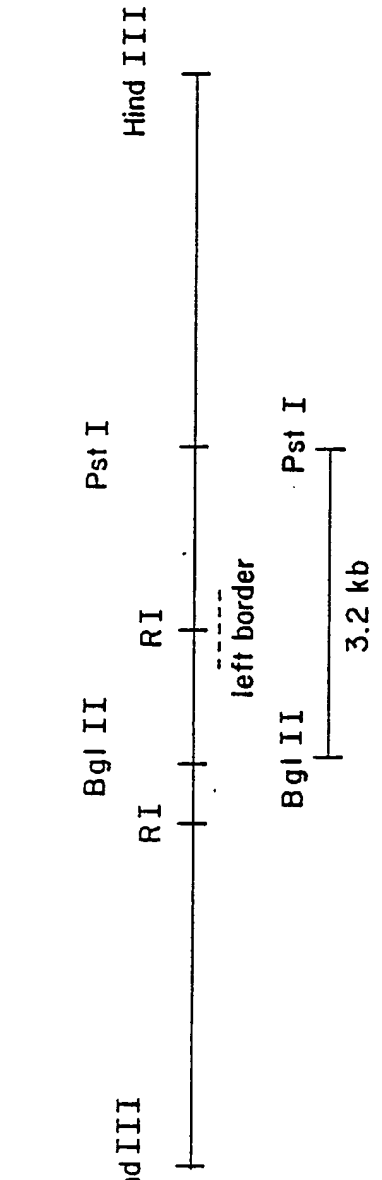


FIG 3
NOS left border

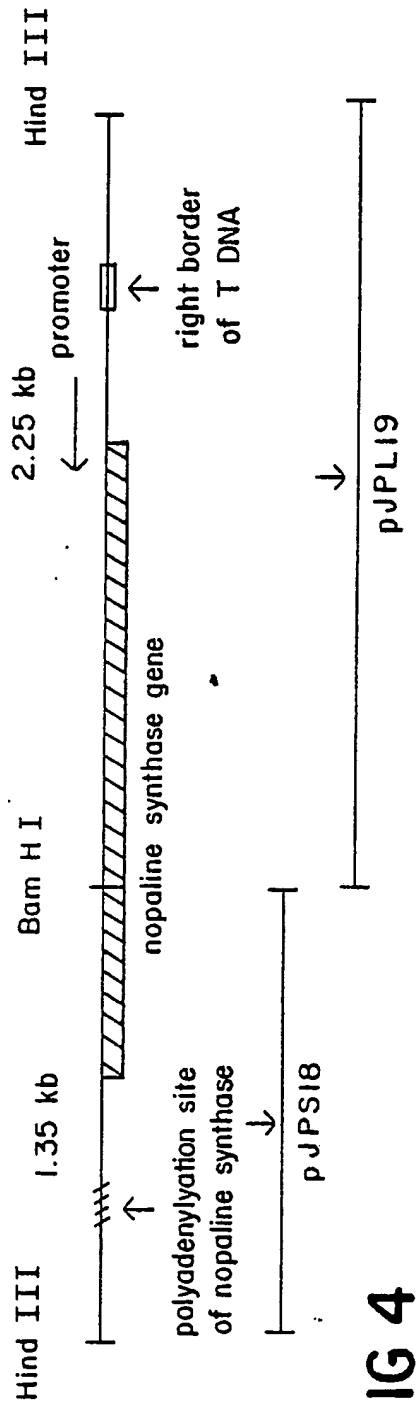


FIG 4

FIG 5

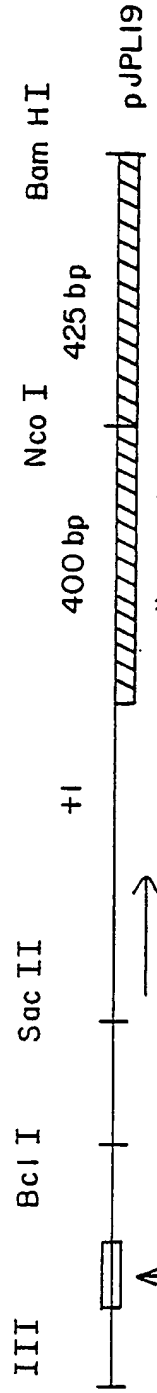
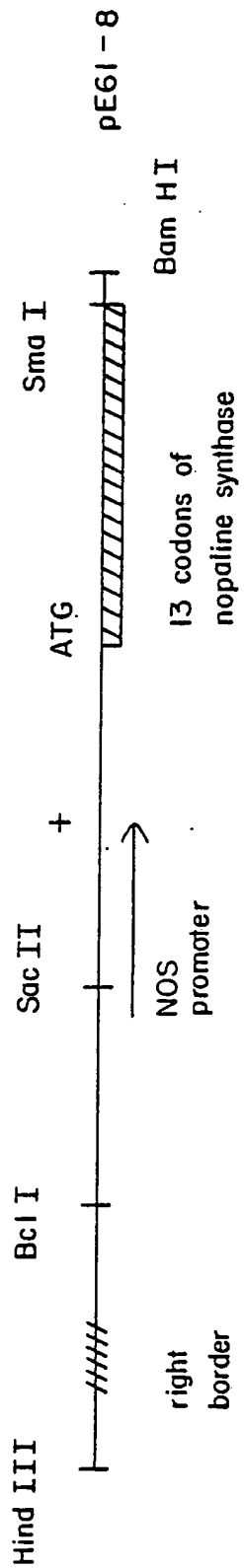

Hind III
Bcl I
Sac II
+1
Nco I
400 bp
425 bp
Bam HI
pJPL19
right border of the nopaline T DNA
NOS promoter
nopaline synthase gene

FIG 6


Hind III
Bcl I
Sac II
+
ATG
Sma I
Bam HI
pE6I-8
right border
NOS promoter
13 codons of nopaline synthase

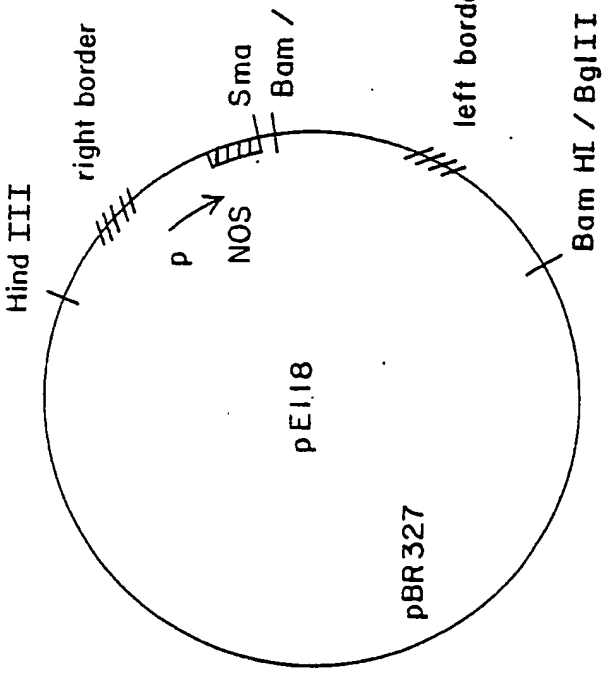

Hind III
right border
p
NOS
Sma
Bam / Pst I
left border
Bam HI / Bgl II
pE118
pBR327

FIG 7

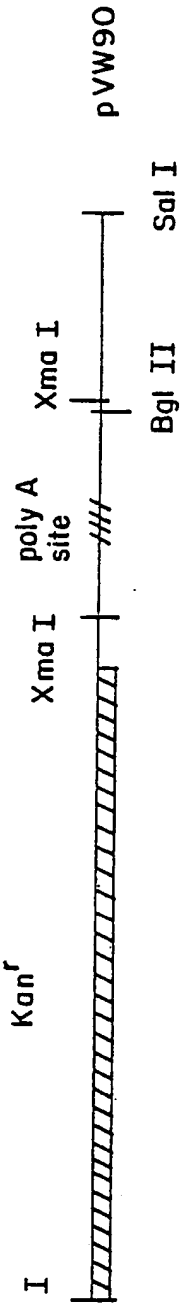

pVW90
Sal I
Bgl II
Xma I
poly A site
Xma I
Kan^r
Sal I

FIG 8

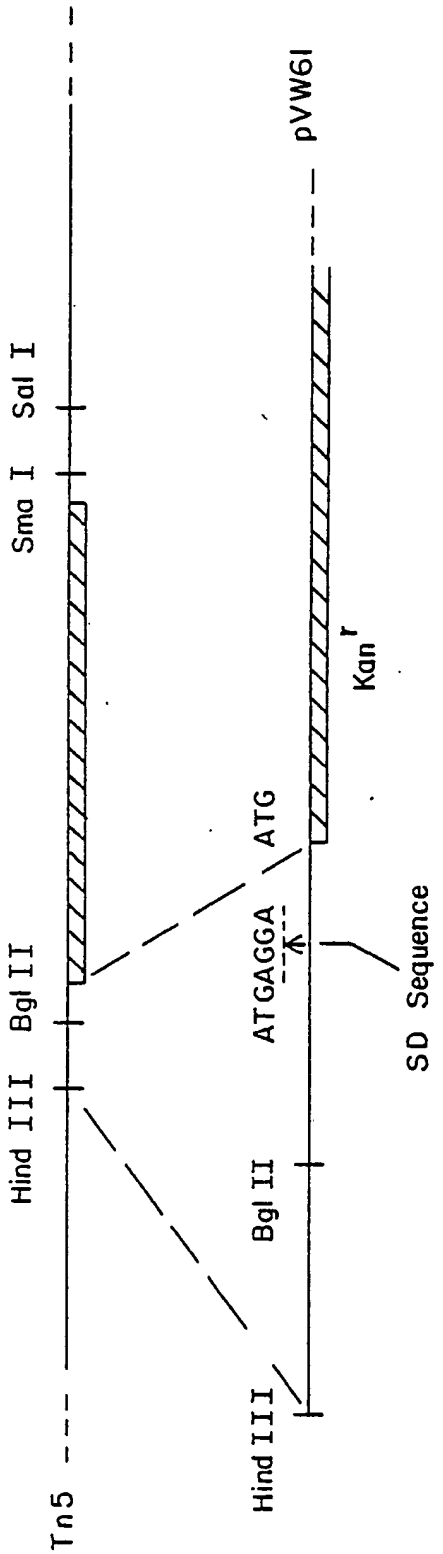

Tn5
Hind III
Bgl II
Sma I
Sal I
pVW61
Kan^r
ATG
ATGAGGA
SD Sequence
Bgl II
Hind III

FIG 9

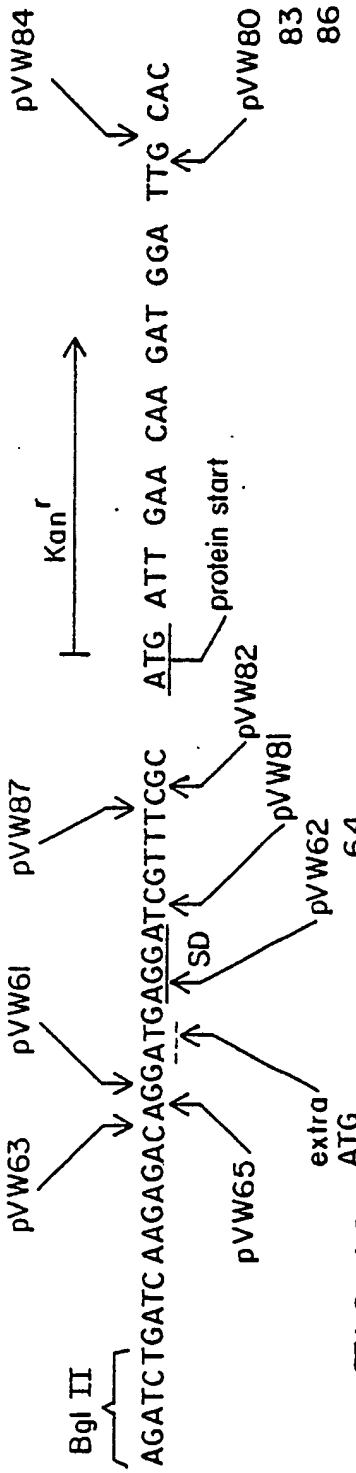

pVW84
pVW80
83
86
Kan^r
ATG ATT GAA CAA GAT GGA TTG CAC
protein start
pVW82
pVW87
pVW81
pVW61
pVW62
64
70
SD
pVW63
pVW65
extra ATG
Bgl II
AGATCTGATC AAGAGACAGGATGAGGATCGTTTCGC

FIG 10

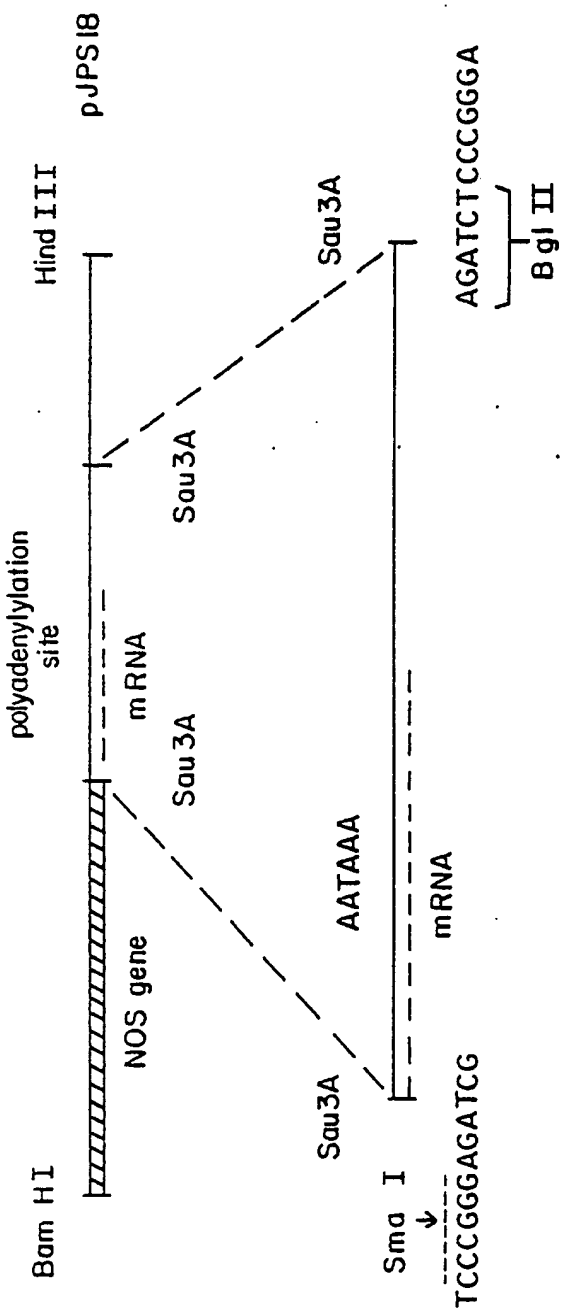

pJPS18
Hind III
Bam HI
polyadenylylation site
mRNA
NOS gene
Sau3A
Sau3A
Sau3A
Sau3A
AATAAA
mRNA
Sma I
TCCCGGGAGATCG
AGATCTCCCGGGA
Bgl II

FIG 11

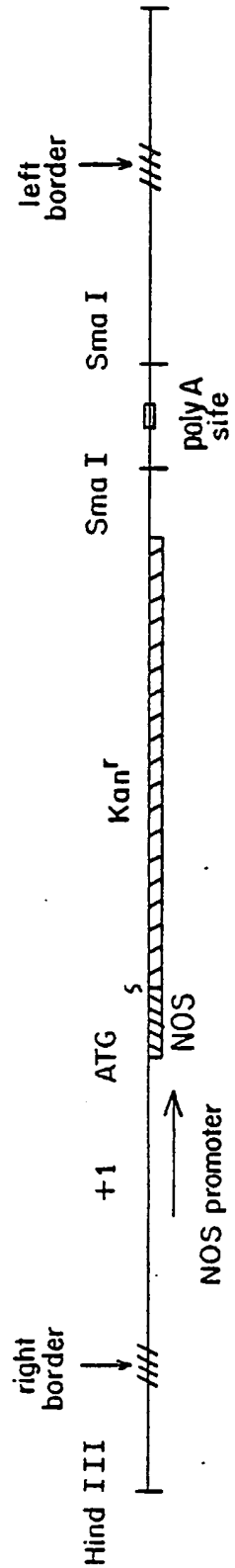

left border
Sma I
Sma I
poly A site
Kan^r
right border
Hind III
+1
ATG
NOS
NOS promoter

FIG 12

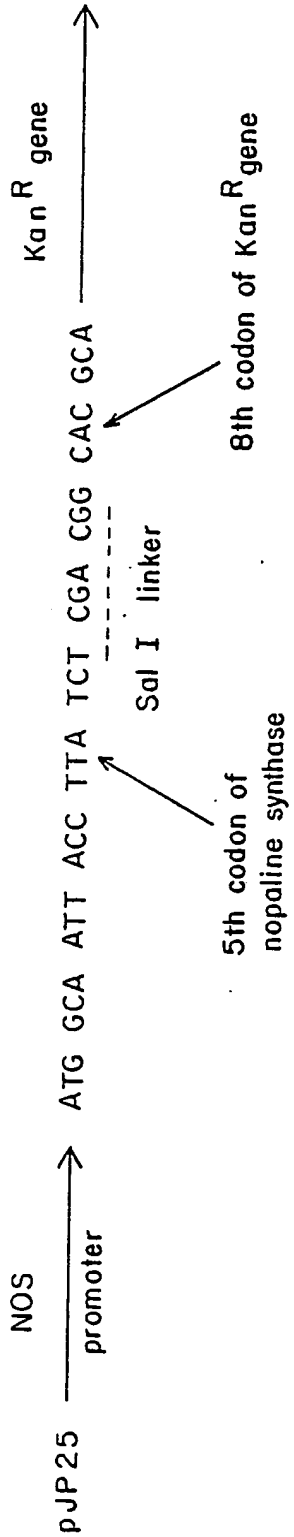

pJP25
NOS
promoter
ATG GCA ATT ACC TTA TCT CGA CGG CAC GCA
Sal I linker
5th codon of nopaline synthase
8th codon of Kan^R gene
Kan^R gene

FIG 13

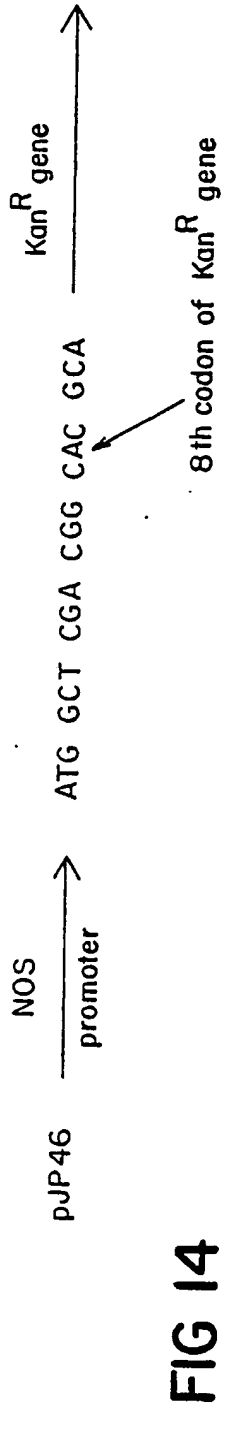

pJP46
NOS
promoter
ATG GCT CGA CGG CAC GCA
8th codon of Kan^R gene
Kan^R gene

FIG 14

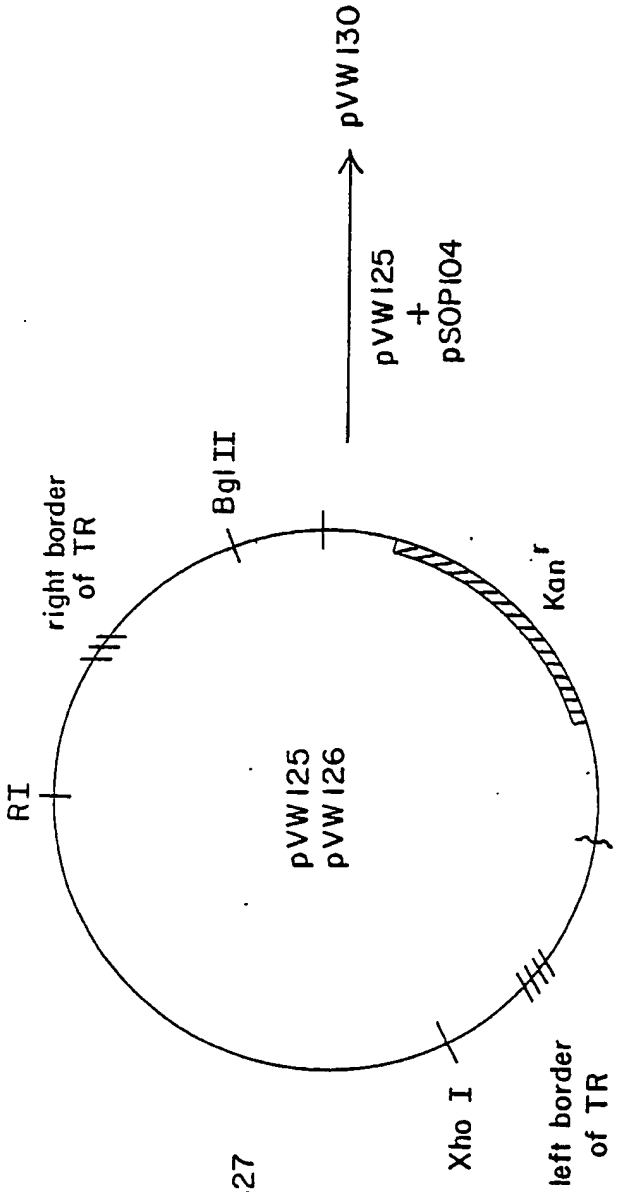

pVW130
pVW125
+
pSOP104
Bgl II
right border
of TR
Kan r
RI
pVW125
pVW126
Xho I
left border
of TR
Vector
pBR 327

FIG 15

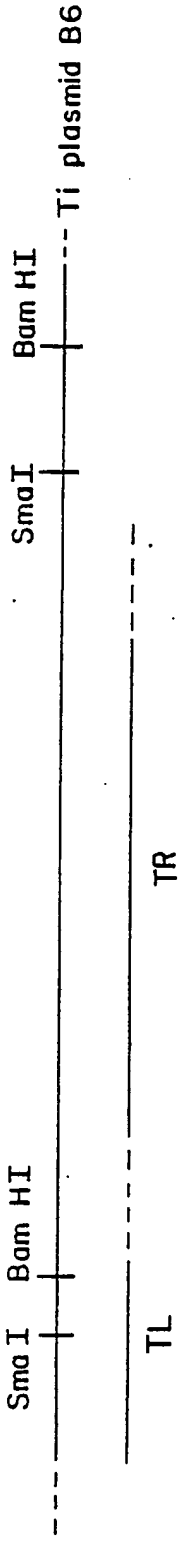
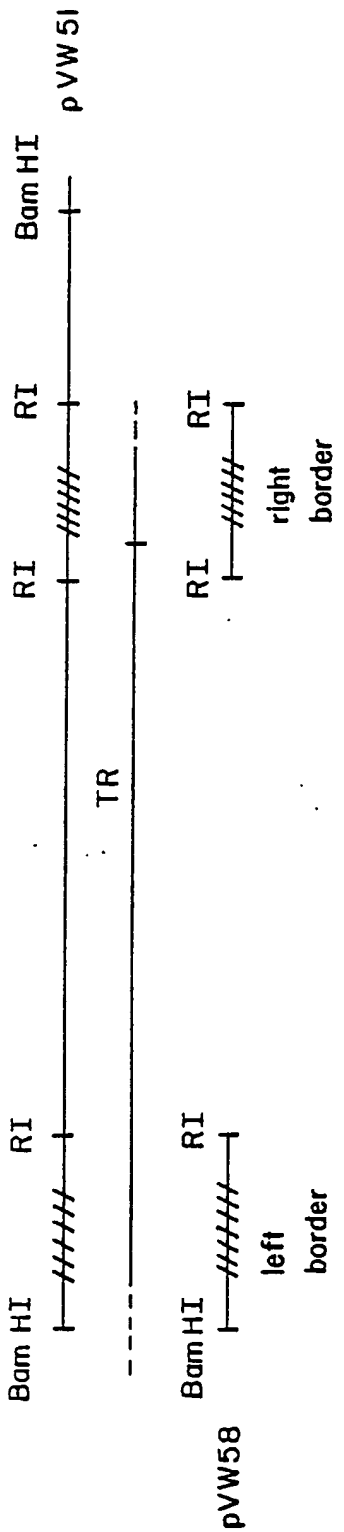
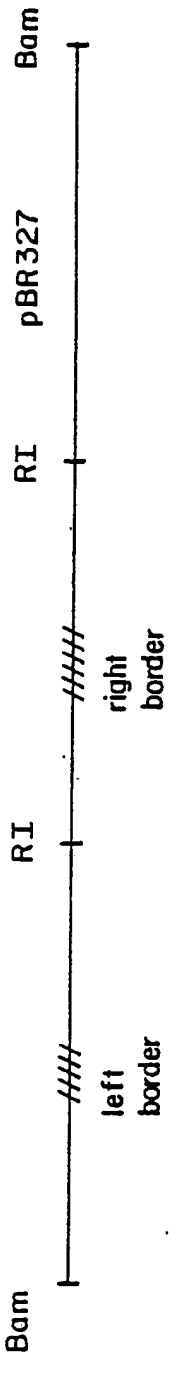

Ti plasmid B6
Bam HI
Sma I
TR
Sma I
Bam HI
TL

FIG 16

FIG 17





This plasmid is pVW75

FIG 18

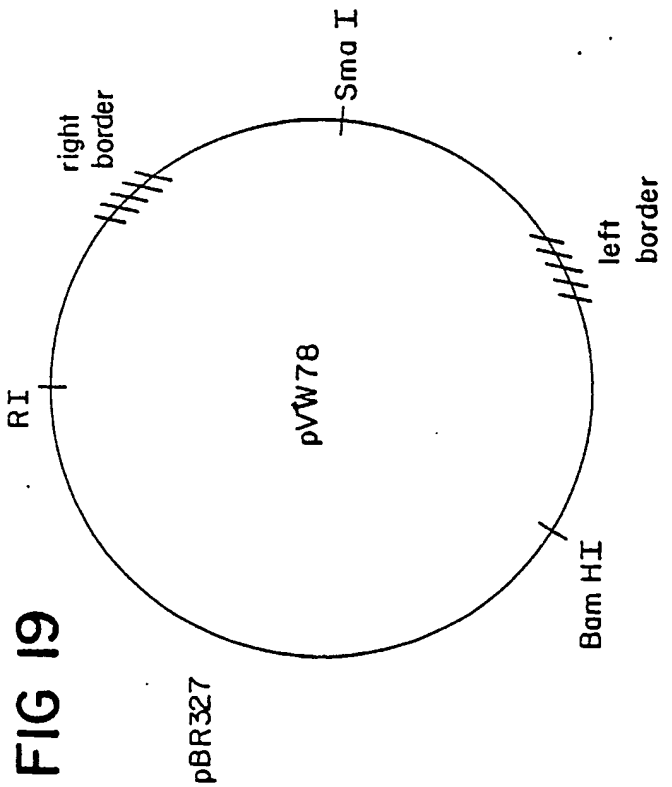

RI
right border
Sma I
left border
pVW78
Bam HI
pBR327

FIG 19

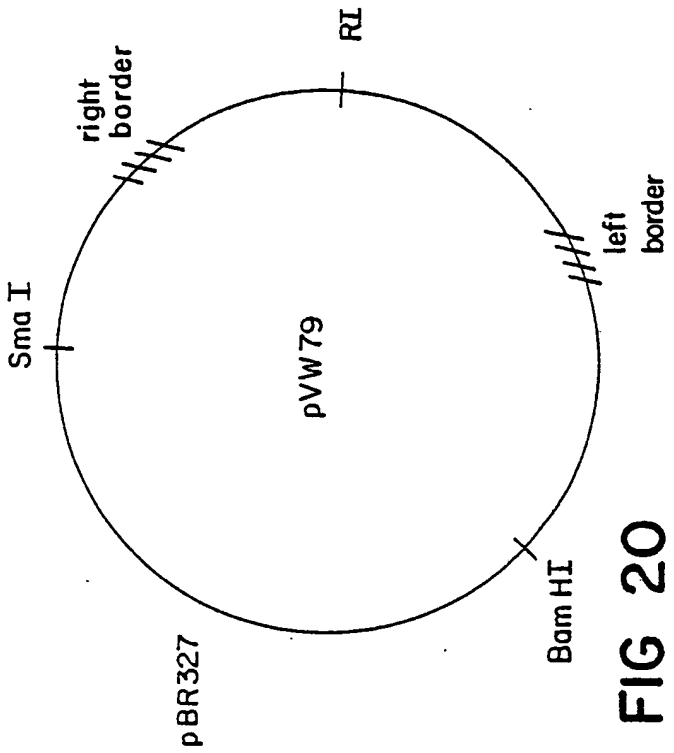

RI
right border
Sma I
left border
pVW79
Bam HI
pBR327

FIG 20

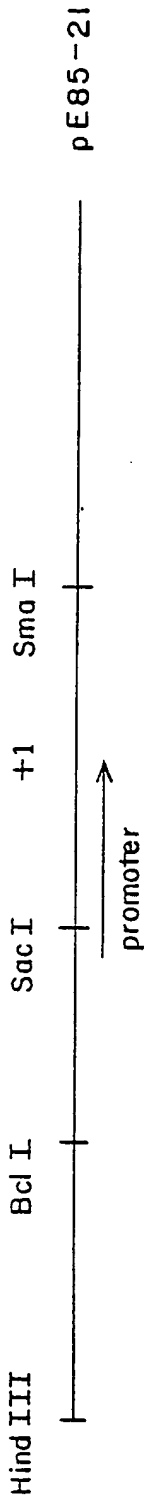

pE85-21
Hind III
Bcl I
Sac I
+1
Sma I
promoter

FIG 21

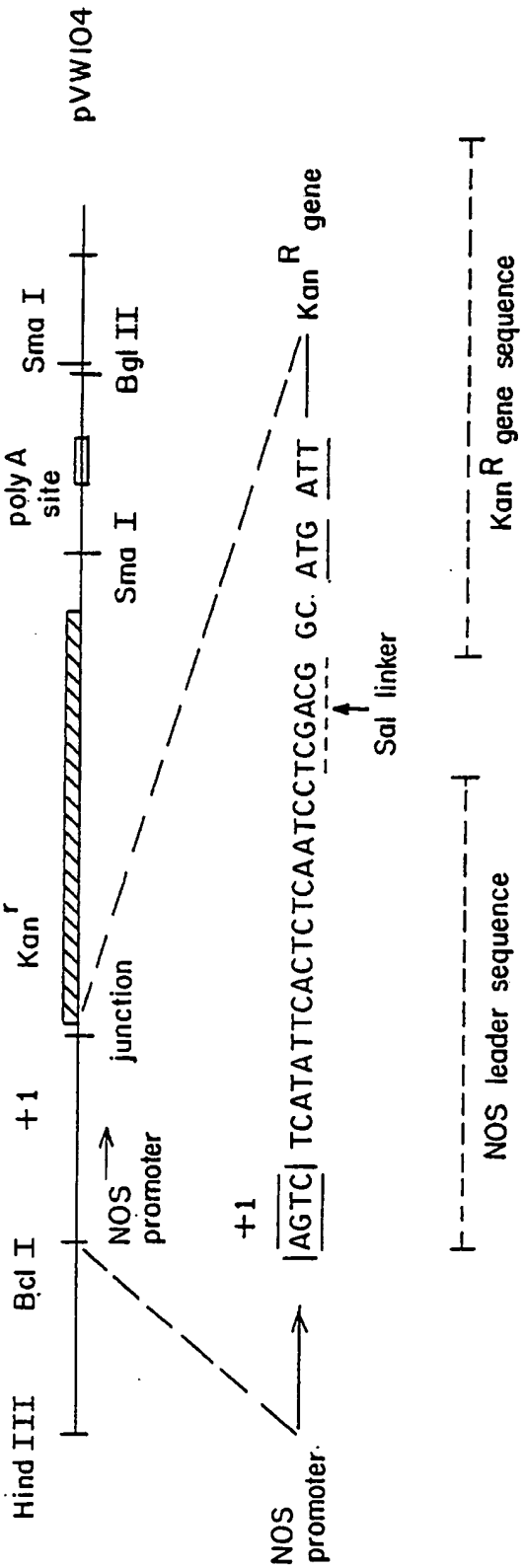

pVW104
Hind III
Bcl I
+1
Kan r
junction
poly A site
Sma I
Bgl II
Sma I
NOS promoter
NOS promoter
+1
AGTC TCATATTCACTCTCAATCCTCGACG GC. ATG ATT
Kan R gene
Sal linker
NOS leader sequence
Kan R gene sequence

FIG 22

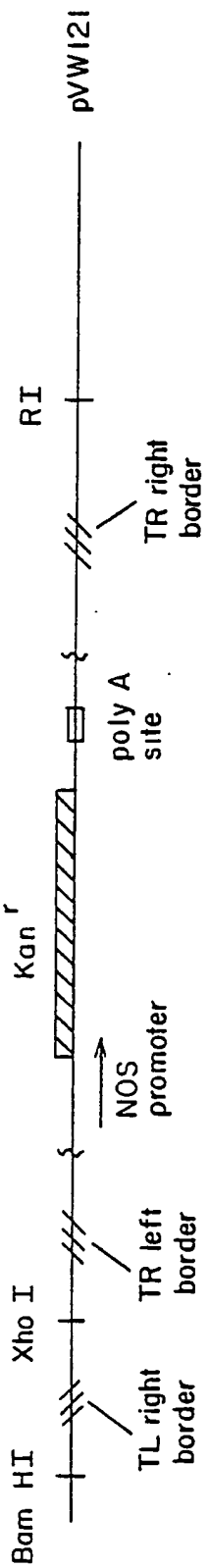

Bam HI
Xho I
TL right border
TR left border
Kan r
NOS promoter
poly A site
TR right border
RI
pVW121

FIG 23

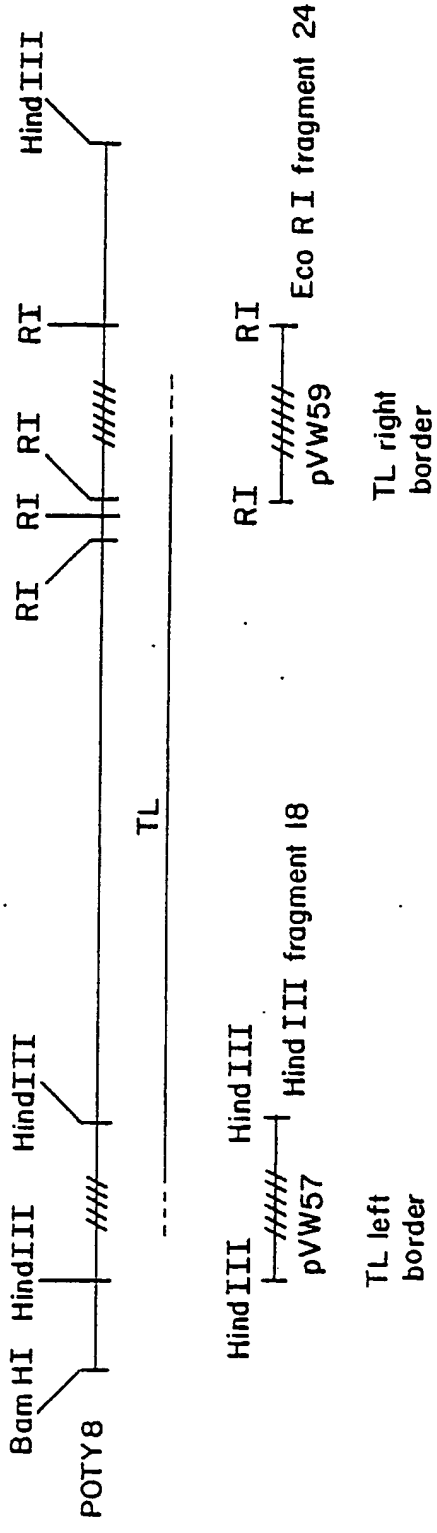
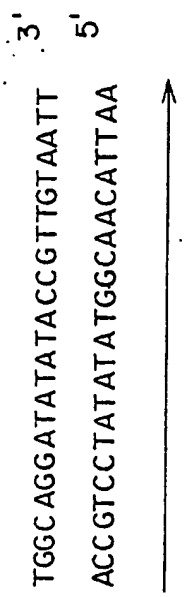

Bam HI
HindIII
POTY8
HindIII
RI
RI
RI
RI
HindIII
TL
HindIII
HindIII fragment 18
pVW57
RI
RI
Eco RI fragment 24
pVW59
TL left border
TL right border

FIG 24

FIG 25

25 bp direct repeat of the octopine TL right border



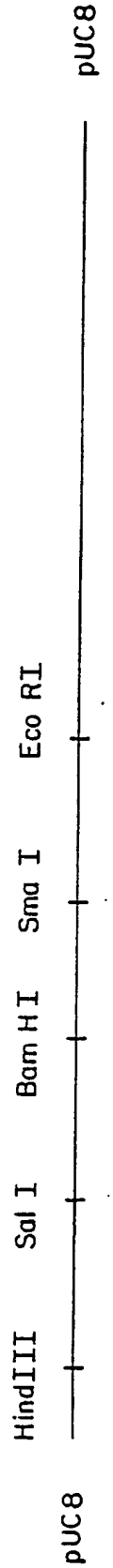


FIG 26

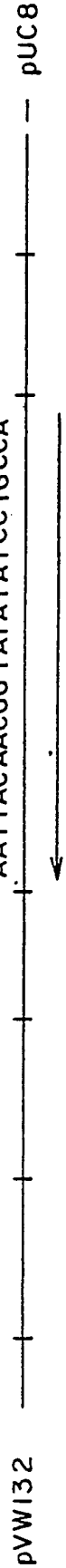

pVW132
Hind III
Sal I
Bam HI
Sma I
Sma I
Eco RI
pUC8
AATTACAACGGTATATATCCTGCCA

FIG 27

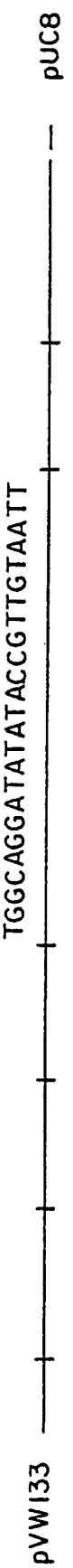

pVW133
Hind III
Sal I
Bam HI
Sma I
Sma I
Eco RI
pUC8
TGGCAGGATATATACCGTTGTAATT

FIG 28

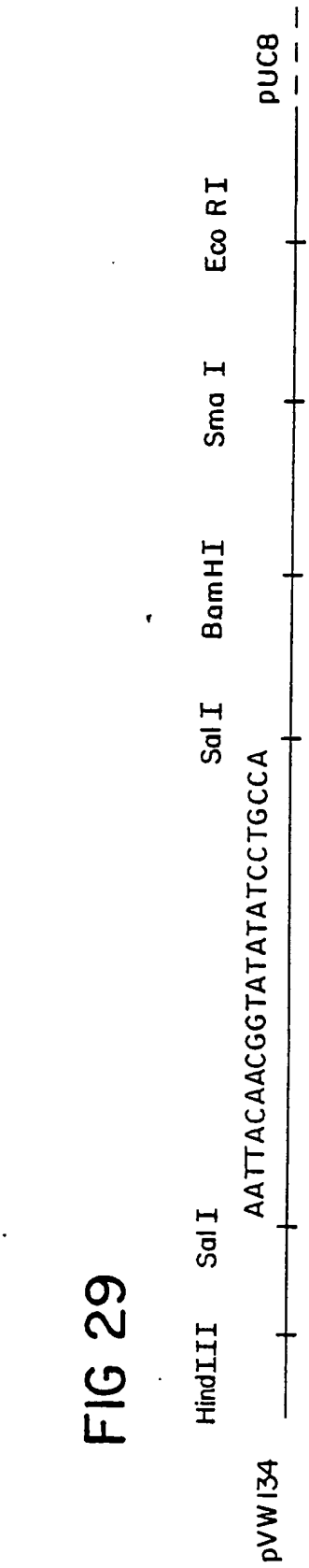

pVW134
HindIII
Sal I
AATTACAACGGTATATATCCTGCCA
Sal I
BamHI
Sma I
Eco RI
pUC8

FIG 29

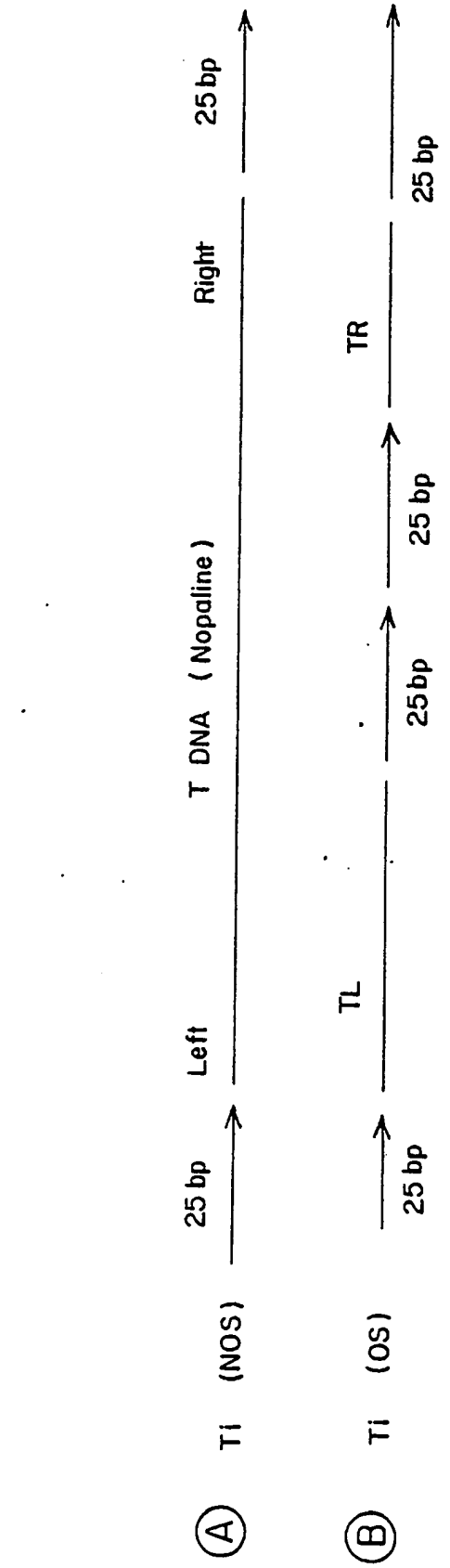

A
Ti (NOS)
25 bp
Left
T DNA (Nopaline)
Right
25 bp
B
Ti (OS)
25 bp
TL
25bp
25 bp
TR
25 bp

FIG 30

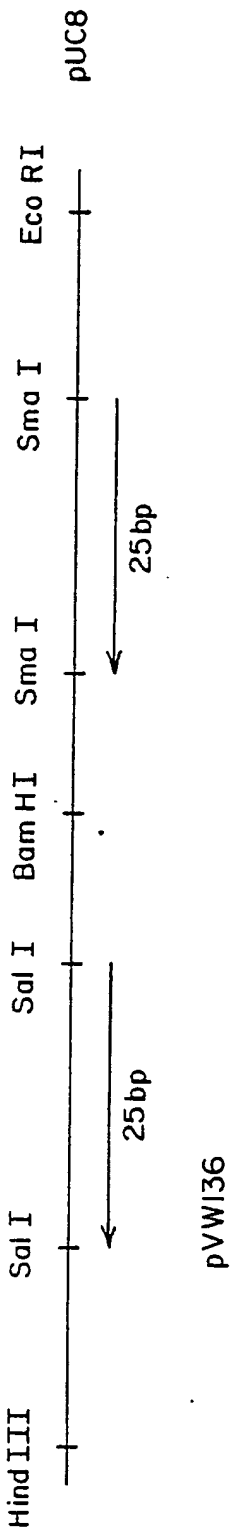

pUC8
Eco RI
Sma I
25bp
Sma I
Bam HI
Sal I
25bp
Sal I
Hind III
pVW136

FIG 31

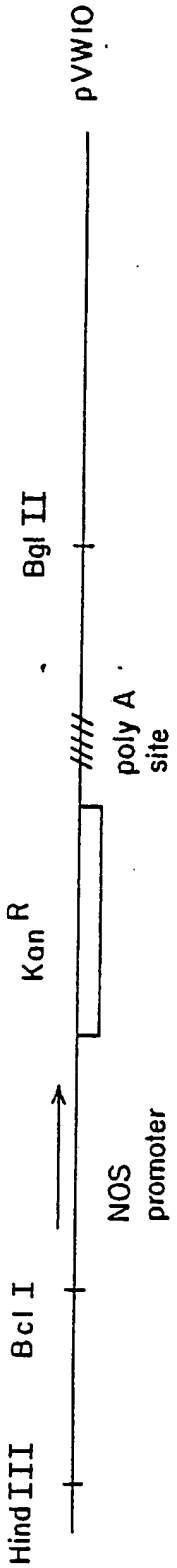

pVW10
Bgl II
poly A site
Kan R
NOS promoter
Bcl I
Hind III

FIG 32

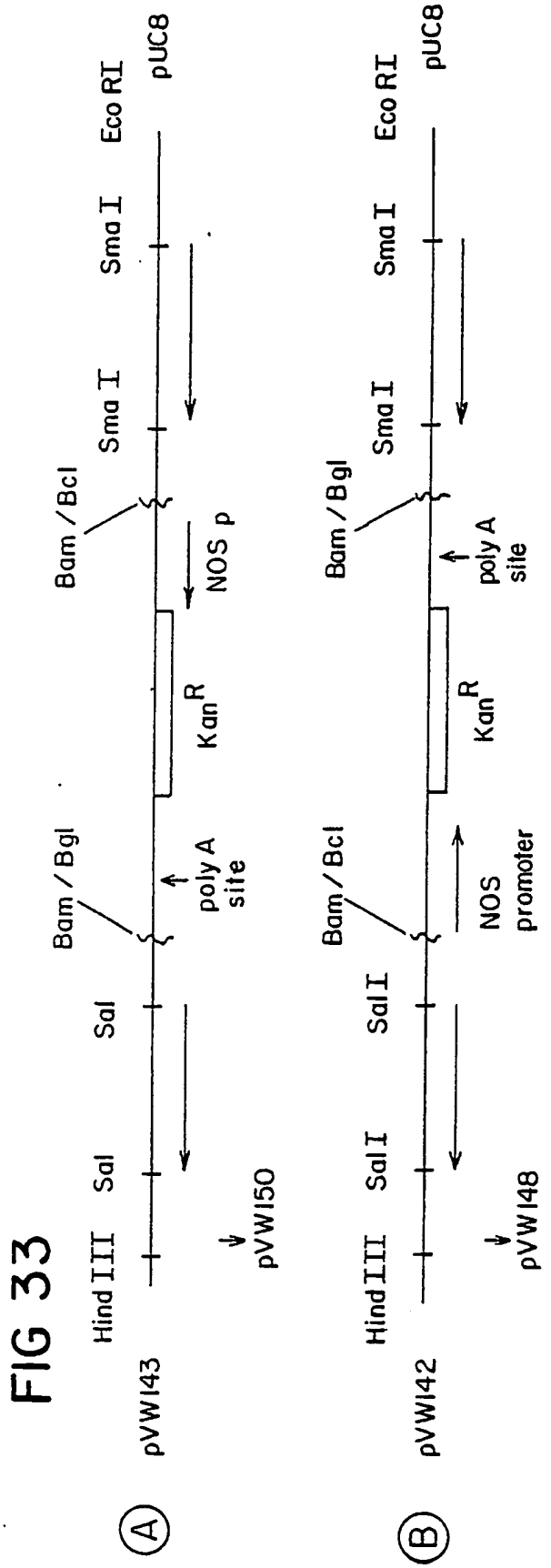

A
pVW143
Hind III
Sal
Sal
Bam / Bgl
poly A site
Kan^R
NOS p
Bam / Bcl
Sma I
Sma I
Eco RI
pUC8
pVW150
B
pVW142
Hind III
Sal I
Sal I
Bam / Bcl
NOS promoter
Kan^R
Bam / Bgl
Sma I
poly A site
Sma I
Eco RI
pUC8
pVW148
pVW140
Hind III
Sal I
Bam H I / Bcl I
NOS promoter
Kan^R
Bam H I / Bgl II
Sma I
poly A site
Sma I
25 bp
Eco RI
pVW144

FIG 33

FIG 34

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02488

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl.[4] C12N 5/00 C12N 15/00 C12N 1/20 C12N 1/00 C07H 21/00
US. Cl. 435/172.3, 240, 241 317

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/172.3, 240, 241, 317

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	N, Bevan et al., 1983, "A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation" Nature Vol. 304 pages 184-187	ALL
X	N, Fraley et al. 1983 "Expression of bacterial genes in plant cells" Proc. Natl. Acad. Sci. Vol. 80 pages 4803-07	ALL
Y	N, Dhaese et al. 1983 "Identification of sequences involved in the polyadenylation of higher plant nuclear transcripts using Agrobacterium T-DNA genes as models" EMBO Journal Vol.2 pages 419-26	1-7
X	N, Krens et al. 1982 "In vitro transformation of plant protoplasts with Ti-plasmid DNA" Nature Vol. 296 pages 72-4	8
Y	N, Herrera-Estrella et al. 1983 "Chimeric genes as dominant selectable markers in plant cells" EMBO Journal Vol. 2 pp 987-95	1-7

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
25 February 1985	11 MAR 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Stephanie Seidman, Ph. D., J.D.

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, Zambryski et al. 1983 "Ti plasmid vector for the introduction of DNA into plant cells without altration of their normal regeneration capacity" EMBO Journal Vol. 2 pages 2143-2150	ALL

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.